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The chaperone function of cyclophilin 40 maps to a cleft between the prolyl isomerase and tetratricopeptide repeat domains

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Abstract **Cyclophilin 40 (CyP40), an immunophilin cochaperone present in steroid receptor-Hsp90 complexes, contains an N-terminal peptidylprolyl isomerase (PPIase) domain separated from a C-terminal Hsp90-binding tetratricopeptide repeat (TPR) domain by a 30-residue linker. To map CyP40 chaperone function, CyP40 deletion mutants were prepared and analysed for chaperone activity. CyP40 fragments containing the PPIase domain plus linker or the linker region and the adjoining TPR domain retained chaperone activity, whilst individually, the catalytic and TPR domains were devoid of chaperoning ability. CyP40 chaperone function then, is localized within the linker that forms a binding cleft with potential to accommodate non-native substrates.**

Keywords: Cyclophilin 40; Peptidylprolyl isomerase; Tetratricopeptide repeat domain; Chaperone activity; Heat shock protein 90

1. Introduction

Cyclophilin 40 (CyP40), a 40 kDa cyclosporin A-binding immunophilin, was initially isolated as a component of estrogen aporeceptor complexes in association with heat shock protein 90 (Hsp90) [1,2]. CyP40 bears sequence and structural homology with other members of the large immunophilin protein family, such as FKBP51 and FKBP52, which bind the immunosuppresant FK506 [3]. These immunophilins are characterised by a binding site for the immunosuppressive drug located in the N-terminal domain, overlapping a peptidylprolyl isomerase (PPIase) function. They also possess a domain of three tetratricopeptide repeat (TPR) units at their C-terminal ends which directs specific protein–protein interactions [4,5].

These TPR-containing immunophilins are present in mature steroid receptor heterocomplexes formed in a step-wise, dynamic assembly process involving the Hsp70 and Hsp90 molecular chaperones and assisted by several cochaperone partners [6]. Hsp90, an abundant molecular chaperone whose association with steroid receptors is critical for hormone binding and signal transduction [6], exists within mature unactivated receptor heterocomplexes as a homodimer. Immunophilins interact directly with Hsp90 through their TPR domain and compete for a common binding site positioned in the C-terminal region of the molecular chaperone [7,8].

Studies of cortisol insensitivity in New World primates revealed lowered hormone binding affinity of the glucocorticoid receptor (GR), attributed to high expression levels of FKBP51 [9]. In a yeast model, FKBP52 selectively potentiated GR transcriptional activity which could be blocked by co-expression of FKBP51 [10]. The immunophilins may therefore have a role in regulating hormone recognition and this is thought to be directed by FKBP52 PPIase activity, which targets specific proline residue(s) in the ligand-binding domain of the receptor [5,10].

In addition to PPIase function and Hsp90 binding, chaperone activity has been reported for CyP40 [11,12], FKBP51 [12] and FKBP52 [11,13], as well as for p23, a partner cochaperone present in mature steroid receptor heterocomplexes [6,14,15]. Specific inhibition of PPIase activity by its cognate immunosuppressant excluded a role for immunophilin catalytic activity in chaperone function [11,13]. Of the two yeast CyP40 homologues, Cpr6 has greater PPIase activity over Cpr7, although Cpr7 is a more potent molecular chaperone [16]. Deletion of Cpr7 resulted in reduced GR-regulated activity [17]. Additional studies suggested that the influence of Cpr7 on GR activity does not involve the PPIase domain, but rather the TPR-containing carboxyl terminus [18].

To gain further insights into the role played by CyP40 in steroid receptor heterocomplexes, we mapped the domain for CyP40 chaperone function. We provide evidence that the chaperone function of CyP40 resides within a hydrophobic cleft between the PPIase and TPR domains and maps specifically to the linker region separating these two domains.

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Abbreviations: CyP40, cyclophilin 40; bCyP40, bovine cyclophilin 40; FKBP*n*, FK506 binding protein of *n* kDa; GR, glucocorticoid receptor; Hsp*n*, heat shock protein of *n* kDa; GST, glutathione *S*-transferase; PPIase, peptidylprolyl isomerase; TPR, tetratricopeptide repeat

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2. Materials and methods

2.1. Expression constructs and purification of GST- and His-CyP40 related proteins

Expression plasmids for glutathione *S*-transferase (GST)-bovine CyP40 (bCyP40) full-length and the deletions 185–370, 1–213 and 1–352 (Fig. 1) have been described previously [19]. For GST-bCyP40 1–185 and 216–370 constructs, DNA encoding these fragments was amplified by PCR from a pGEX2T-bCyP40 full-length template and cloned into the pGEX4T.1 and pGEX2T vectors (Amersham Biosciences), respectively. Similarly, the His-bCyP40 1–184, 1–215 and 1–235 constructs were prepared by PCR amplification using the pGEX2T-bCyP40 full-length template and cloned into the pET28a(+) vector (Novagen). Generation of GST-bCyP40 185–370Δ243–256 was accomplished by PCR using pGEM-3Z-bCyP40 1–370 as a template [19] and oligonucleotide primers incorporating *Bal*I (5′ end) and *Sma*I (3′ end) restriction sites. A *Bal*I site existing at amino acid position 242 in bCyP40 was designed into the forward primer immediately preceding the nucleotide sequence for amino acid positions 257–264. The PCR product was subjected to *Bal*I and *Sma*I digestion and ligated into pGEX2T-bCyP40 185-370 previously digested with *Bal*I and *Sma*I. The pET28a(+)-hHsp90β 527–724 plasmid for His-hHsp90β 527–724 protein expression has been previously described [20].

Plasmids for bacterial expression of GST and GST- and His-CyP40 fusion proteins were transformed into *E. coli* BL21 (codon +) cells and were purified according to previously described methods [20]. Recovered protein eluates were pooled, then concentrated and equilibrated with chaperone assay buffer (50 mM HEPES, pH 7.0, 100 mM NaCl) in a Centricon YM-30 (Millipore, Bedford, MA) before storage at −70 °C. GST-bCyP40 216–370 gave a low recovery and was therefore dialysed against chaperone assay buffer using Slide-A-Lyzer dialysis cassettes (Perbio Science, Cheshire, UK). Protein concentration was determined by the Bradford assay [21].

2.2. Pull-down assays

All constructs generated previously had been assessed for binding to Hsp90 [19]. The GST-bCyP40 216–370 and 185–370Δ243–256 constructs (0.4 μM) were incubated with purified His-hHsp90β 527–724 (1.8 μM) and subjected to a pull-down assay as described previously [22]. After SDS–PAGE, resolved proteins were visualized by Coomassie staining and Hsp90 binding status was further evaluated by Western blot analysis with AC88 antibody [23] to detect Hsp90.

2.3. Circular dichroism spectroscopy

His-tagged bCyP40 fragments were dialysed in 50 mM potassium phosphate, pH 7.4 and diluted to 0.033 mg/ml protein in the same buffer for analysis. Far UV CD measurements were performed in a Jasco

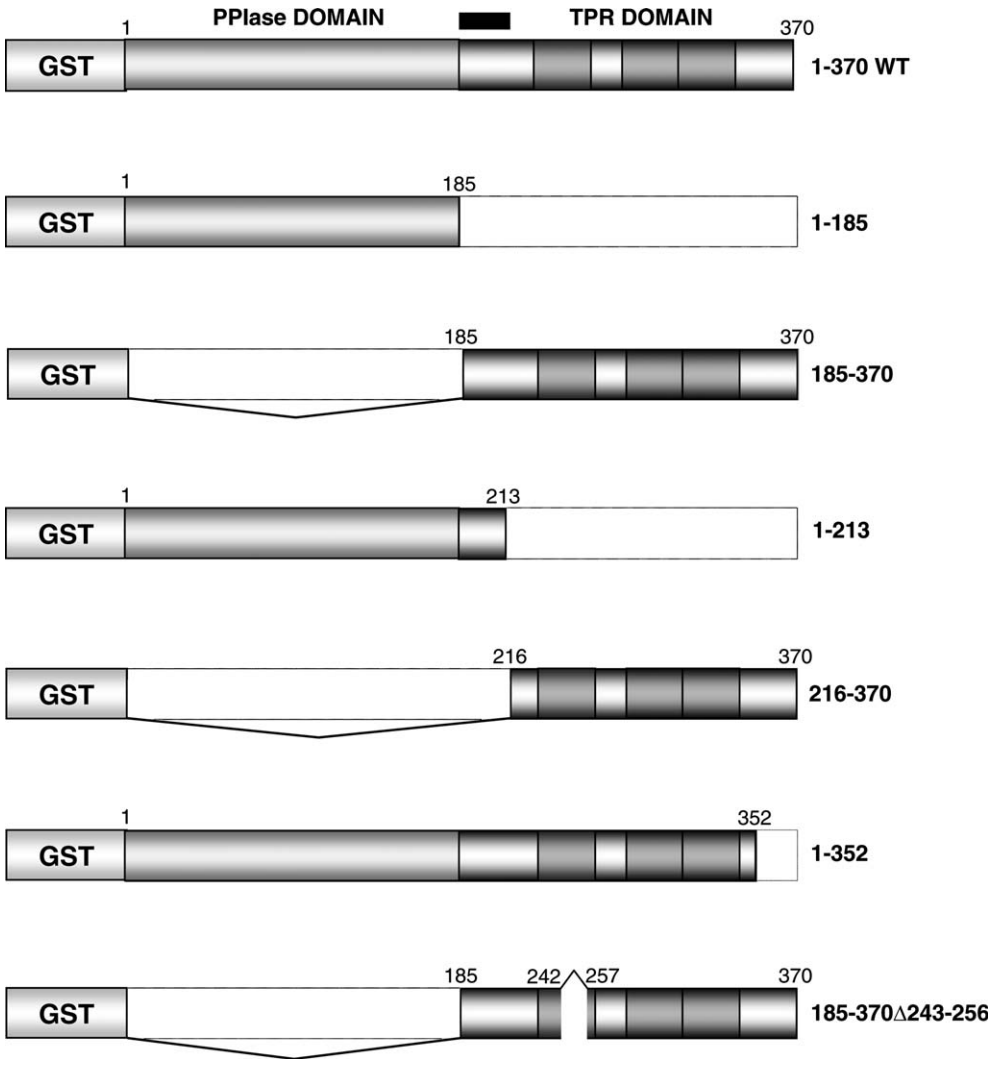

Fig. 1. Schematic of GST-bCyP40 wild-type (WT) fusion protein and deletion constructs. The light shaded box depicts the PPIase domain and the dark boxes represent units of the TPR domain. The solid bar indicates the linker region. Numbers indicate CyP40 amino acid positions. The caret shown in the last construct represents deleted amino acids. The GST moiety is not to scale.

810 spectropolarimeter at a constant temperature of 25 °C. Spectra were measured from 250 to 190 nm in a 1 cm path length quartz cuvette and recorded as an average over 50 accumulations. Secondary structure was assessed using the CDSSTR program available from the DICHROWEB website [24,25].

2.4. Chaperone assays

An aggregation assay, using rhodanese as the substrate, was adapted from published methods [8,26] and has been previously described [20]. The GST- and His-bCyP40 fusion, and GST control proteins were analysed at concentrations of 0, 5, 10 and in some cases, 20 μM, in chaperone assay buffer with denatured substrate (0.5 μM) and the rate of rhodanese aggregation monitored over 20 min. Each protein was checked for self-aggregation over a 5-min period.

3. Results

3.1. CyP40 chaperone activity lies in the C-terminal half of the protein and does not require an intact PPIase domain

Bovine CyP40 is characterized by a two-domain structure in which the N-terminal catalytic domain is separated from the C-terminal TPR protein interaction domain via a 30-residue linker [19,27]. The chaperone function of the human CyP40 homologue has previously been demonstrated with the GST-tagged recombinant protein [11,12]. To map the location of the CyP40 chaperone domain we assessed the chaperone function of several GST fusion proteins incorporating bCyP40 deletion fragments (Fig. 1) using an aggregation assay with rhodanese [26,28,29]. The extent of rhodanese aggregation in the presence of GST-bCyP40 proteins is shown in Figs. 2 and 3.

Rhodanese aggregation was suppressible by GST-bCyP40 1–370 wild-type protein in a concentration-dependent manner (Fig. 2A). At 20:1 chaperone:substrate molar ratio, the full-length protein reduced rhodanese aggregation by approximately 45%. In control experiments, inhibition of rhodanese aggregation was not evident at any concentration of GST protein tested (Fig. 2D). We observed no chaperone activity for the 1–185 construct, corresponding to the isolated PPIase domain (Fig. 2B). However, the C-terminal 185–370 fragment, incorporating the linker and TPR domain, retained chaperone function. This deletion mutant decreased the level of rhodanese aggregation by approximately 42% at 10 μM concentra-

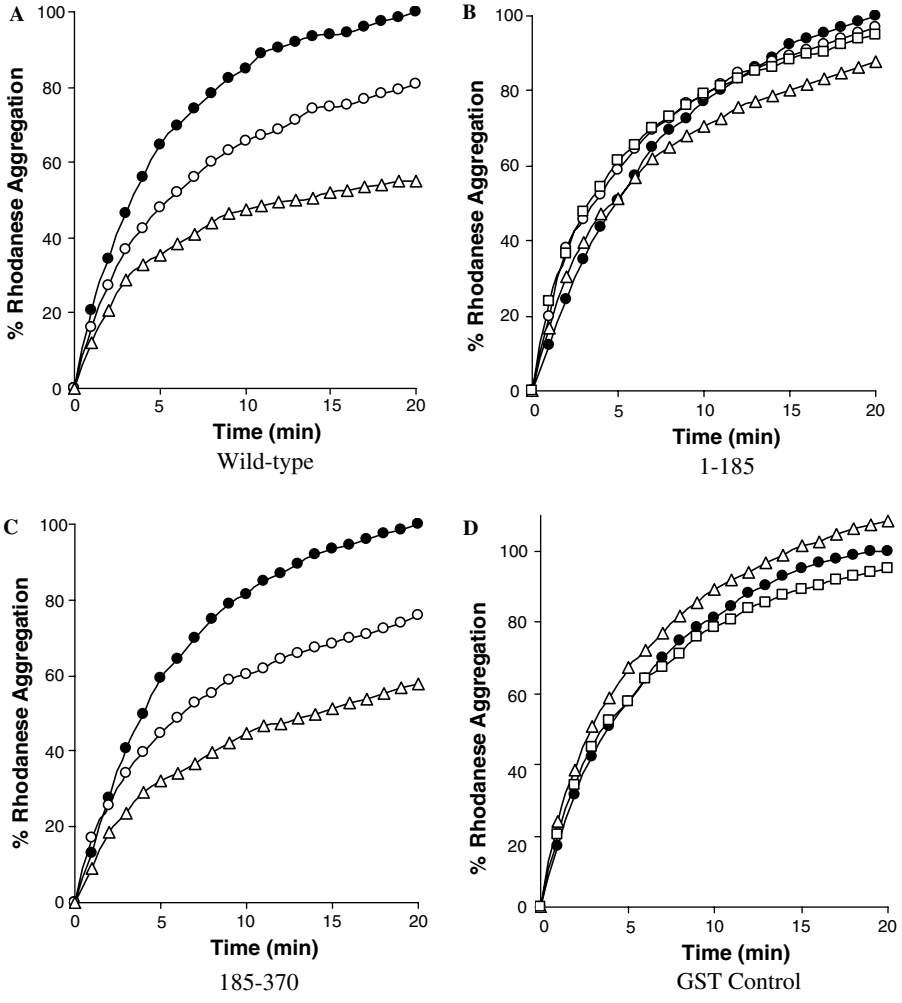

Fig. 2. Broad mapping of chaperone function in bovine CyP40. Denatured rhodanese was diluted in assay buffer to 0.5 μM in the absence (●) or presence of 5 μM (○), 10 μM (△) or 20 μM (□) GST control or GST-CyP40 fusion protein. Rhodanese aggregation was monitored at 320 nm and absorbance normalised against runs with rhodanese alone and plotted as percent aggregation over a 20-min period. The effect on rhodanese aggregation is shown for: (A) GST-bCyP40 wild-type; (B) GST-bCyP40 1–185; (C) GST-bCyP40 185–370; and (D) GST.

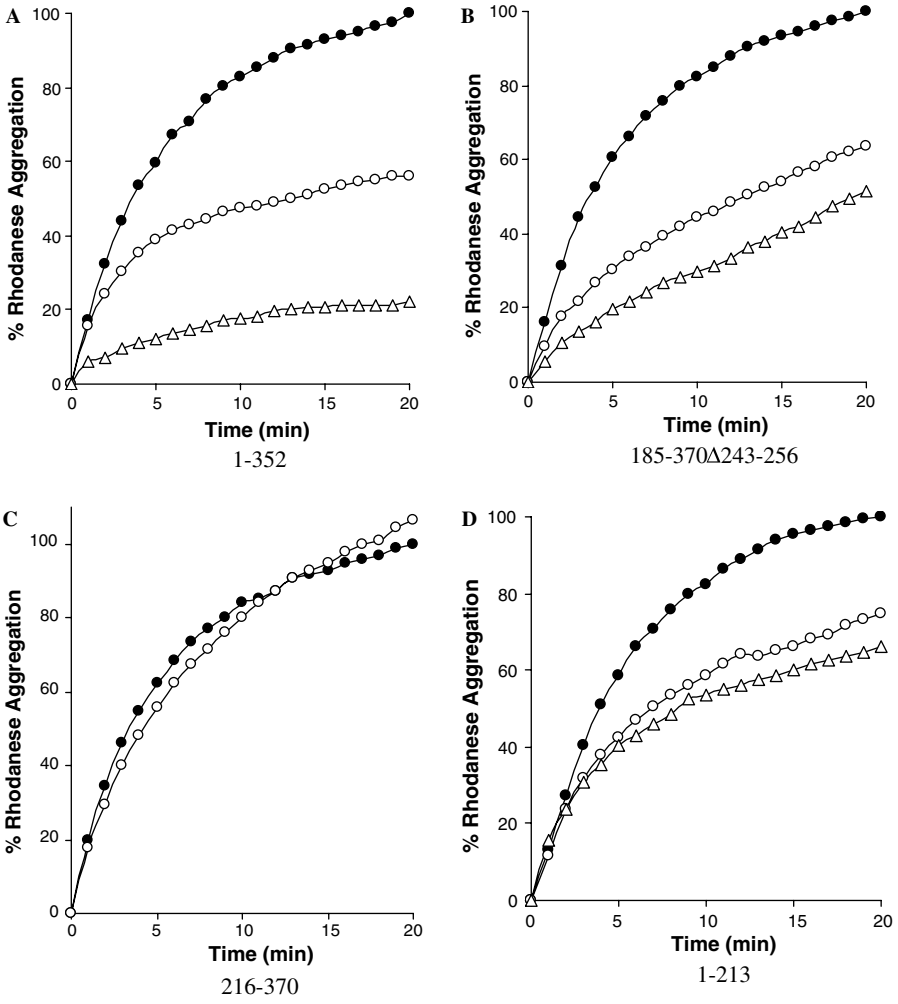

Fig. 3. Fine mapping of chaperone function in bovine CyP40. Experimental conditions are as in Fig. 2. Rhodanese aggregation was monitored in the absence (●) or presence of 5 μM (○) or 10 μM (△) GST-CyP40 fusion protein. The effect on rhodanese aggregation is shown for: (A) GST-bCyP40 1–352, (B) GST-bCyP40 185–370Δ243–256, (C) GST-bCyP40 216-370 and (D) GST-bCyP40 1–213.

tion (Fig. 2C), a result comparable to that observed for full-length CyP40.

3.2. Distinct elements in CyP40 determine chaperone function and Hsp90 interaction

Since our evidence suggested CyP40 chaperone function resides within the C-terminal half of the protein, we next investigated whether the TPR domain, which is essential for CyP40–Hsp90 interaction, plays a role in this activity. As part of our experimental approach we assessed the chaperone function of deletion mutants that abrogated immunophilin binding to Hsp90, allowing us to determine whether there was an overlap between these two functional domains within CyP40 or whether they were distinct. The final helix of the CyP40 structure (helix V, corresponding to residues 341–370) extends the TPR domain [27] and contains the so-called charge-Y motif [30] that contributes significantly to CyP40–Hsp90 interaction [20]. Deletion of 18-residues from the C-terminus of CyP40 in the 1–352 construct, sharply reduced binding to Hsp90 compared to full-length protein [19]. Intriguingly, the 1–352 fragment showed an increased chaperoning ability over wild-type CyP40, with an ~80% reduction of rhodanese aggregation at 20-fold excess of chaperone protein (Fig. 3A). It is possible that this deletion relieved steric constraints to favor more efficient chaperone function. One cannot rule out, however, that these changes may have exposed other features in the CyP40 protein resulting in increased chaperone potency.

To further evaluate the TPR domain for chaperone function, we created the 185–370Δ243–256 construct in which most of helix Q in the first TPR unit [27] has been eliminated. With such a gross disruption of TPR domain structural integrity, this deletion mutant was not able to bind Hsp90 (Fig. 4), but the protein still displayed a chaperone function similar to the 185–370 construct (Fig. 3B, Fig. 2C). Although the first TPR unit in CyP40, based on the canonical 34-residue motif, begins at Ser223, structural data shows that the first helix (helix P) of the TPR domain is longer, beginning with Val216 [27]. We therefore prepared the 216–370 fragment that lacks the linker region, but retains an intact TPR domain. This protein displayed a markedly reduced level of Hsp90 binding in comparison to that seen with GST-CyP40 1–370 (Fig. 4), consistent with our previous finding that the CyP40 linker may contribute to Hsp90 interaction [19]. Fig. 3C shows that the 216–370 fragment did not prevent rhodanese aggregation at 10-fold molar

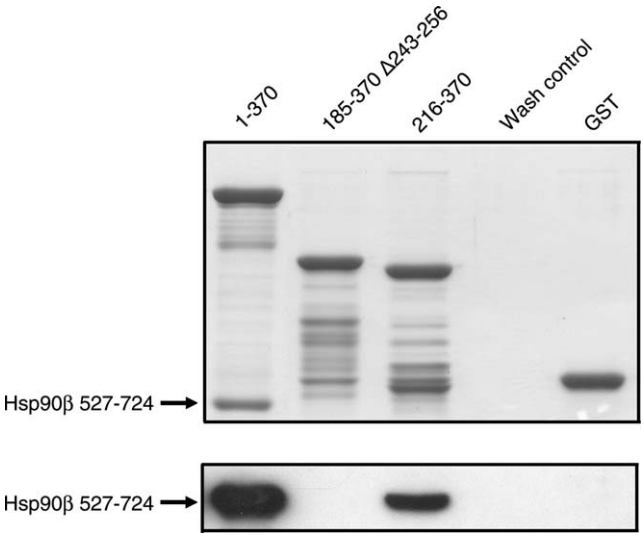

Fig. 4. Binding of CyP40 deletion mutants to Hsp90. Purified GST-bCyP40 1–370 wild-type or mutant fusion proteins were incubated with purified His-hHsp90β 527–724 and then bound to glutathione-Sepharose beads. For the "wash control", the Hsp90 fragment was incubated with glutathione-Sepharose beads alone. After washing, proteins retained on the beads were resolved on duplicate 12.5% SDS–polyacrylamide gels and either visualized by Coomassie staining (top panel) or subjected to Western blot with AC88 to detect Hsp90 bound to CyP40 constructs (bottom panel).

excess. Higher concentrations could not be tested, as the TPR domain fragment was prone to self-aggregation.

Our data suggested that neither the PPIase nor the TPR domain mediate CyP40 chaperone activity. We therefore considered that the linker separating the two domains might fulfill this role. Indeed, analysis of the 1–213 construct, containing an intact cyclophilin domain and almost the entire linker region (Fig. 1), confirmed chaperone activity for the protein, although to a diminished extent compared to wild-type CyP40 (Fig. 3D). Only a 34% reduction in rhodanese aggregation was observed at 20:1 molar ratio of chaperone to substrate. Although the linker may also contribute to efficient CyP40–Hsp90 interaction, the key residues for Hsp90 binding are located within the core TPR domain [22]. Taken together, these results map the chaperone domain in CyP40 to the linker region between amino acid residues 185–215 and confirm distinct locations for the chaperone and Hsp90 binding functions of the immunophilin.

Further verification that the linker region mediates chaperone function was provided using His-tagged fusion proteins for bCyP40 1–184, 1–215 and 1–235 (Fig. 5A), the latter fragment containing the first helix of the TPR domain [27]. Secondary structure was determined for these proteins by CD spectroscopy. All three fragments displayed minima indicative of α-helix and β-structure and these results are summarised in Table 1. As a comparison, the secondary structure content for these fragments, based on the available crystal data for bCyP40 [31], is also shown. Overall, the secondary structure of the His-tagged purified fragments of bCyP40 correlates well with its crystal structure.

Analysis of the His-bCyP40 deletion mutants for chaperone function revealed a lack of activity for the 1–184 fragment (Fig. 5A), consistent with our earlier finding for GST-bCyP40 1–185 (Fig. 2B). The His-bCyP40 1–215 and 1–235 fragments however, displayed chaperone properties, with observed reductions in rhodanese aggregation of 30% and 25%, respectively, at 10 μM concentration (Fig. 5B and C). These findings were comparable to that seen for the related construct, GST-bCyP40 1–213 (Fig. 3D).

3.3. A hydrophobic cavity within the linker of CyP40 may mediate chaperone function

Having delineated CyP40 chaperone function to the linker between the PPIase and TPR domains, we examined the intervening sequence for structural features that might favour substrate interaction. In the intact CyP40 protein, the 30-residue linker is structured through well-defined hydrogen bonds and contains 11 aspartate and glutamate residues with connecting salt bridges between residues Asp200 and Asp204 and helix Q of the TPR domain [27]. Ten hydrophobic residues are interspersed throughout the linker, six being located between 200 and 215 (Fig. 6A). A further five hydrophobic amino acids exist at the start of helix P from residues 216 to 222. In comparison to its yeast homologues, Cpr6 and Cpr7, human CyP40 displays similar chaperone activity to Cpr7, but is a more potent chaperone than Cpr6 [12,16]. Alignment of the linker sequences for bovine and human CyP40 with those of Cpr6 and Cpr7, showed some conservation among acidic and hydrophobic residues (Fig. 6A). Close examination of the CyP40 structure within the linker region showed that the side chains of the hydrophobic amino acids Phe205, Pro206, Val211 and Leu213 contribute to a hydrophobic surface within a cleft that is bounded on one side by the PPIase domain and on the other by the first unit of the TPR domain (Fig. 6B). Side chains from the hydrophobic residues Ile219 and Ile222, in helix P of the TPR domain and the amphipathic Tyr252, located within helix Q, all project into this cleft (Fig. 6B). The hydrophobic surface may serve as a potential protein–protein interaction site to protect peptide substrates from aggregation.

4. Discussion

Using specifically designed bovine CyP40-derived deletion mutants, coupled with a rhodanese aggregation assay, we have located the chaperone domain of this immunophilin to the 30-residue linker separating the PPIase and TPR domains. Examination of CyP40 structure has revealed a hydrophobic cleft, within this linker region, that might be central to the ability of the chaperone to accommodate and stabilize unfolded or partially folded substrates. Our initial studies showed retention of wild-type chaperone activity by the C-terminal half of the protein (185–370), thus excluding a role for PPIase activity in CyP40 chaperone function. The result is consistent with an earlier finding, in which cyclosporin A did not affect the ability of CyP40 to maintain β-galactosidase in an intermediary folded state [11] and that cyclophilin domains generally, lack chaperone activity [32]. Chaperone functions independent of PPIase activity have also been reported for other prolyl isomerases, including FKBP52 [13,33] and trigger factor [34].

The C-terminal half of CyP40, which is comprised of the linker region and TPR domain and determines CyP40 interaction with Hsp90, was further dissected for chaperone func-

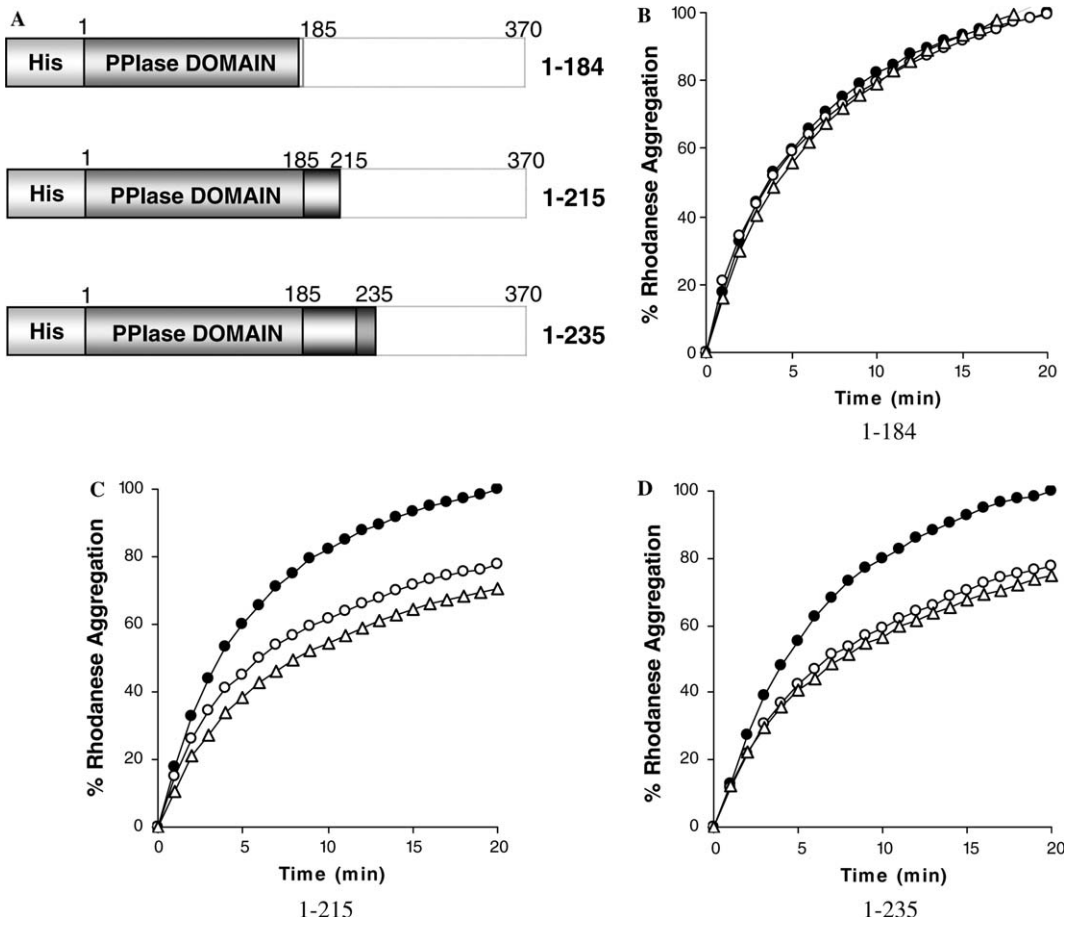

Fig. 5. The CyP40 linker mediates chaperone function. (A) Schematic of His-bCyP40 fusion proteins for 1–184, 1–215 and 1–235. The shading pattern is identical to that described in Fig. 1. The His tag is not to scale. These proteins were tested for chaperone function using experimental conditions as in Fig. 2. Rhodanese aggregation was monitored in the absence (●) or presence of 5 μM (○) or 10 μM (△) GST-CyP40 fusion protein. The effect on rhodanese aggregation is shown for: (B) His-bCyP40 1–184; (C) His-bCyP40 1–215; and (D) His-bCyP40 1–235.

Table 1
Structural properties of His-bCyP40 fragments

bCyP40 Fragment	α-Helix		β-Structure		NRMSD
	CD-derived (%)	Structure-derived (%)	CD-derived (%)	Structure-derived (%)	
1–184	14	11	37	28	0.035
1–215	12	9	33	24	0.049
1–235	16	17	30	22	0.068

Far-UV CD data were recorded and analysed using the CDSSTR program found on the DICHROWEB website (http://www.cryst.bbk.ac.uk/cdweb/html/). The NRMSD values were determined by fitting the spectral data using proteins from Reference set 4. Actual secondary structure content was calculated from the X-ray structure of bCyP40 [34].

tion. While the 216–370 fragment, containing only the TPR domain, was able to bind Hsp90, it was devoid of chaperone activity. On the other hand, CyP40 185–370Δ243–256, which contains a disrupted TPR domain thus precluding Hsp90 interaction, displayed wild-type chaperone activity. Retention of chaperone function by GST-bCyP40 1–213 and His-bCyP40 1–215 proteins, allowed us to conclude that the chaperone domain resides within the linker residues 185–215. Furthermore, there was a clear separation of the CyP40 domains that mediate chaperone activity and Hsp90 recognition. For FKBP52, the chaperone domain has been localized to a region encompassing the TPR domain [33]. There is evidence however, that intrinsic chaperone function and Hsp90 interaction through the TPR domain occur at different sites in FKBP52 [33]. Similarly, an unstructured C-terminal region in the Hsp90 cochaperone, p23, has been shown to be essential for its chaperone function, but is not involved in Hsp90 binding [14,15,35].

Within the CyP40 linker region we have identified a hydrophobic surface formed by amino acid residues Phe205, Pro206, Val211 and Leu213, and the TPR helical residues Ile219, Ile222 and Tyr252, that may provide a substrate-binding cavity for CyP40 chaperone function. The strand incorporating residues 200–212 may close up against the TPR helices, forming the essential arm of a hydrophobic clamp around potential peptide substrates, with TPR residues stabilizing

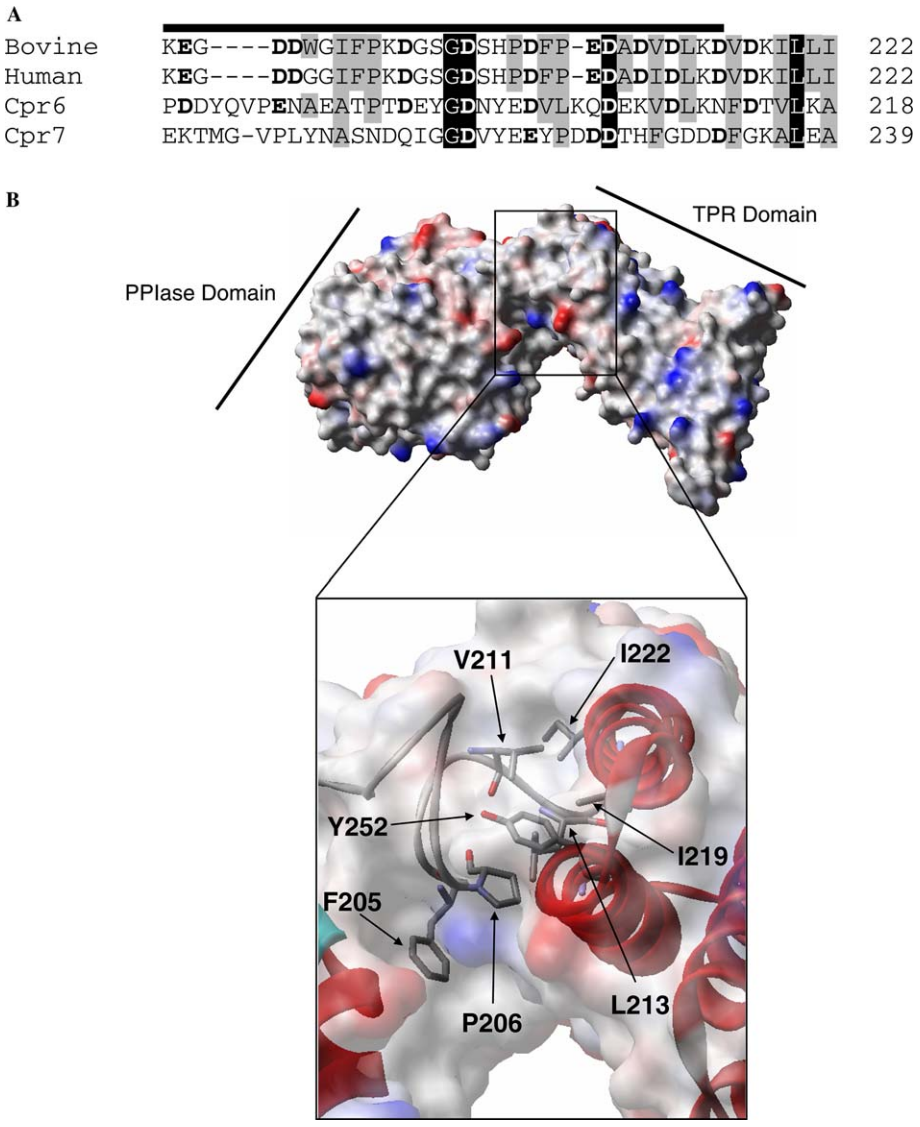

Fig. 6. The chaperone domain of CyP40 resides in a hydrophobic cavity within the linker region. (A) Sequence alignment of amino acids within the linker (line above sequence) and part of the first helix of the TPR domain of bovine CyP40 and its human and two yeast homologues, Cpr6 and Cpr7. Acidic residues conserved with bCyP40 are indicated in bold. Conserved hydrophobic residues are boxed in grey. Residues identical among all four immunophilins are boxed in black. (B) Solvent surface representation of bCyP40 highlighting a putative client protein binding cleft as shown within the box. A magnified view of the linker region is shown below the solvent surface representation highlighting hydrophobic residues proposed to be involved in folding interactions with client proteins.

the interaction. Although not specifically defined, a hydrophobic patch, located within or close to the TPR domain, has been proposed to mediate interactions of non-native substrate proteins with FKBP52 [33]. Specific aspartate and glutamate residues within the CyP40 linker may help stabilize the structure of the hydrophobic cavity and may also influence substrate association. Selected acidic and hydrophobic residues are partially conserved between the linker sequences for human and bovine CyP40 and the yeast homologues, Cpr6 and Cpr7, which display weak and potent chaperone activity, respectively [16].

Within mature unactivated steroid receptor complexes, the cooperative actions of multiple chaperone proteins hold the receptor in a partially misfolded, and thus transcriptionally repressed, conformation in readiness for activating hormonal signal [4,5]. Dynamic receptor assembly and competing influences of the immunophilins with respect to Hsp90 binding, together with immunophilin selectivity for specific receptors, allows receptors to form distinct receptor-Hsp90-immunophilin complexes, with receptor function being determined by the immunophilin within the complex [4,5]. There is now accumulating evidence for the direct interaction of immunophilins with steroid receptors [5,10]. Additionally, there is growing recognition that the interaction between Hsp90 and the immunophilins is not limited to the C-terminal MEEVD recognition motif for TPR immunophilins, but may also involve elements within the Hsp90 dimerization domain [36]. It is possible therefore, for the target(s) of TPR immunophilin chaperone function within steroid receptor complexes to be either receptor and/or Hsp90. Either of these interactions may help achieve an optimal conformation for receptor activation.

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